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Evolution of discocephalid ciliates: Molecular, morphological and ontogenetic data support a sister group of discocephalids and pseudoamphisiellids (Protozoa, Ciliophora) with establishment of a new suborder Pseudoamphisiellina subord. n.

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Discocephalids and pseudoamphisiellids are possibly two of the most confused groups among hypotrichous/euplotid ciliates regarding their systematic position and phylogenetic relationships. The former were often regarded as related to euplotids while the latter, in the absence of molecular data, were mostly assigned to the urostylid-like hypotrichs. In the present work, the small subunit rRNA genes of several rarely observed discocephalid and pseudoamphisiellid genera were analyzed to obtain insights into the phylogenetic relationships of these highly ambiguous Spirotrichea. Four different tree reconstruction algorithms yielded nearly identical topologies, which indicated both groups belong to the same assemblage. This assemblage is clearly isolated as a deep-branching clade and invariably positioned between Euplotida and Hypotricha. The sister group relationship of the Pseudoamphisiellidae and Discocephalidae supports the previous suggestion that they might represent an ordinal taxon, the Discocephalida. Both morphological and morphogenetic features indicate that the pseudoamphisiellids should be placed in the order Discocephalida but as a sister group to other typical discocephalids. Thus we propose establishing a new suborder, Pseudoamphisiellina subord. n. The new taxon is diagnosed by the following characteristics: (i) two distantly separated midventral rows that are morphogenetically formed with an urostylid mode; (ii) absence of the "frontoterminal row", which is formed from the posterior-most frontoventral-transverse cirral anlage in all other typical urostylids; (iii) numerous caudal cirri that derive from each of the dorsal kinety anlagen; (iv) right marginal row that has a unique *de novo* origin; and (v) inhabiting periphytic communities. The validity of the suborder Pseudoamphisiellina is firmly supported by molecular data.

Ciliophora, Discocephalida, Pseudoamphisiellina subord. n., new suborder, phylogeny, SSU rRNA gene

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The class Spirotrichea is one of the most diverse groups of ciliated protozoa inhabiting marine biotopes. The morphological and morphogenetic diversity of spirotrichs, together with their ecological importance, make them the most studies of all ciliates. Two groups of hypotrich ciliates, discocephalids and pseudoamphisiellids, characterized by a unique combination of many morphological and morphogenetic features, are extremely problematic in their placement in the class Spirotrichea (*sensu* Lynn 2008) or in hypotrichs (*sensu* Berger 2006). The discocephalids have been repeatedly recognized as a euplotid group [1–5], whereas pesudoamphisiellids are always considered a highly specialized hypotrichous taxon [2,3,6–8]. The systematic positions of these two groups have not yet been rigorously verified as

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only two genera, *Prodiscocephalus* and *Pseudoamphisiella*, have been investigated genetically based on small subunit (SSU) rRNA gene sequences [7–9].

In the present study, we focused on these two groups and used all molecular information available, including two genera of discocephalids (*Paradiscocephalus* and *Discocephalus*) and one genus of the family Pseudoamphisiellidae (*Leptoamphisiella*). Our primary goal was to study the relationships and the phylogenetic placement of these taxa. The findings will provide important insights into the origin and evolution of Spirotrichea, especially the morphologically diverse euplotids and stichotrichs. Moreover, we evaluated the potential utility of SSU rRNA gene sequences for future phylogenetic studies of spirotrichs.

1 Materials and methods

1.1 Samples, DNA extraction, and amplification and sequencing of SSU rRNA genes

Samples of *Leptoamphisiella vermis* were collected from the coast of Qingdao (36°04′N, 120°20′E). Organisms were isolated and identified according to published descriptions [10]. Terminology and classification systems followed Berger [11,12].

Total genomic DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St Louis, USA). SSU rRNA coding regions were amplified by PCR according to Miao *et al.* [13], using two primers complementary to the 5′ and 3′ termini of eukaryotic 16S-like rRNA genes [14]. The full-length PCR products were purified by agarose gel electrophoresis, cloned in the pUCm-T vector (Sangon, Toronto, ON, Canada), and sequenced on both strands by the Takara sequencing facility, Shanghai, China.

1.2 Alignment and phylogenetic analysis

Complete or nearly complete SSU rRNA gene sequences in this study were obtained from the GenBank/EMBL database and aligned manually using BioEdit software [15]. MrModeltest 2 [16] was used to identify the optimal evolutionary model (a general time-reversible model), and showed that among-site rate variation was best modeled with gamma correction (=0.4631) [17] and invariant sites (=0.2993) [18]. A maximum likelihood (ML) analysis was performed using the software PHYML 2.4.4 [19]. For maximum parsimony (MP) analysis in PAUP* 4.0b10 [20], a tree bisection and reconnection (TBR) heuristic search was used with a parsimony ratchet with all characters equally weighted and unordered. Statistical support for the ML and MP analyses was obtained from 1000 bootstrap replicates. The program MrBayes 3.0b4 [21] was used to construct a Bayesian tree using Markov chain Monte Carlo algorithm. The chain length for our analysis was 10000000 generations with trees sampled every 100 generations; the first 25% were discarded as burn-in. Posterior probabilities (PP) at nodes were estimated from the remaining trees. A cladogram was constructed using the neighbor-joining (NJ) method [22] with the Kimura two-parameter model [23] using the PHYLIP 3.66 package [24]. TreeView 1.6.6 [25] and MEGA 4.0 [26] were used to visualize tree topologies. Finally, a comparison of the likelihood of the best topology with the likelihood of the candidate topologies (shown below) was performed with the approximately unbiased (AU) test [27] implemented in PAUP* 4.0b10 [20]. The test determines whether the alignment shows significant conflict with the favored topology.

2 Results

2.1 Sequence comparison

The target SSU rRNA nucleotide sequence of *Leptoamphisiella vermis* is 1776 bp in length (GenBank accession No. FJ865203). We compared the complete SSU rRNA gene sequence among discocephalids and pseudoamphisiellids. *Paradiscocephalus elongatus* and *Prodiscocephalus borrori* showed 36 bp differences in the primary structure of the SSU rRNA gene, a number that is much less than the differences between *P. elongatus* and *Discocephalus ehrenbergi* (154 bp), and between *D. ehrenbergi* and *P. borrori* (154 bp). Closely related species in the genus *Pseudoamphisiella* showed sequence differences of 6–103 bp, while *L. vermis* differed from *Pseudoamphisiella* species by 56–88 bp.

2.2 Alignment and SSU rRNA gene genealogy

Base composition across the entire data set was fairly uniform (26.5% A, 19.8% C, 26.1% G, and 27.6% T), and no evidence of saturation was found in the sequences used (data not shown). All major nodes supported by partitioned bootstrap analyses were in agreement.

The pseudoamphisiellid-discocephalid clade was an isolated assemblage, though with alternative affinities (hypotrichs) in all four topologies (MP, ML, BI, and NJ) (data not shown). Sister-grouping of the pseudoamphisiellids and discocephalids was moderately supported by BI posterior probabilities (0.92), but was poorly supported by MP (bootstrap support 56%) and equivocal in the ML and NJ analysis (Figures 1 and 2). Consistently, *Discocephalus* was sister to *Prodiscocephalus* and *Paradiscocephalus*, (Figures 1 and 2). *Pseudoamphisiella lacazei* was more closely related to *L. vermis* (support values: 53% ML, 65% MP, 1.00 BI, 64% NJ) than to other *Pseudoamphisiella* species.

Using the AU test, we tested the likelihood of the following five topologies against the best ML topology: (i) hypotrichs (including the family Urostylidae) are mono-

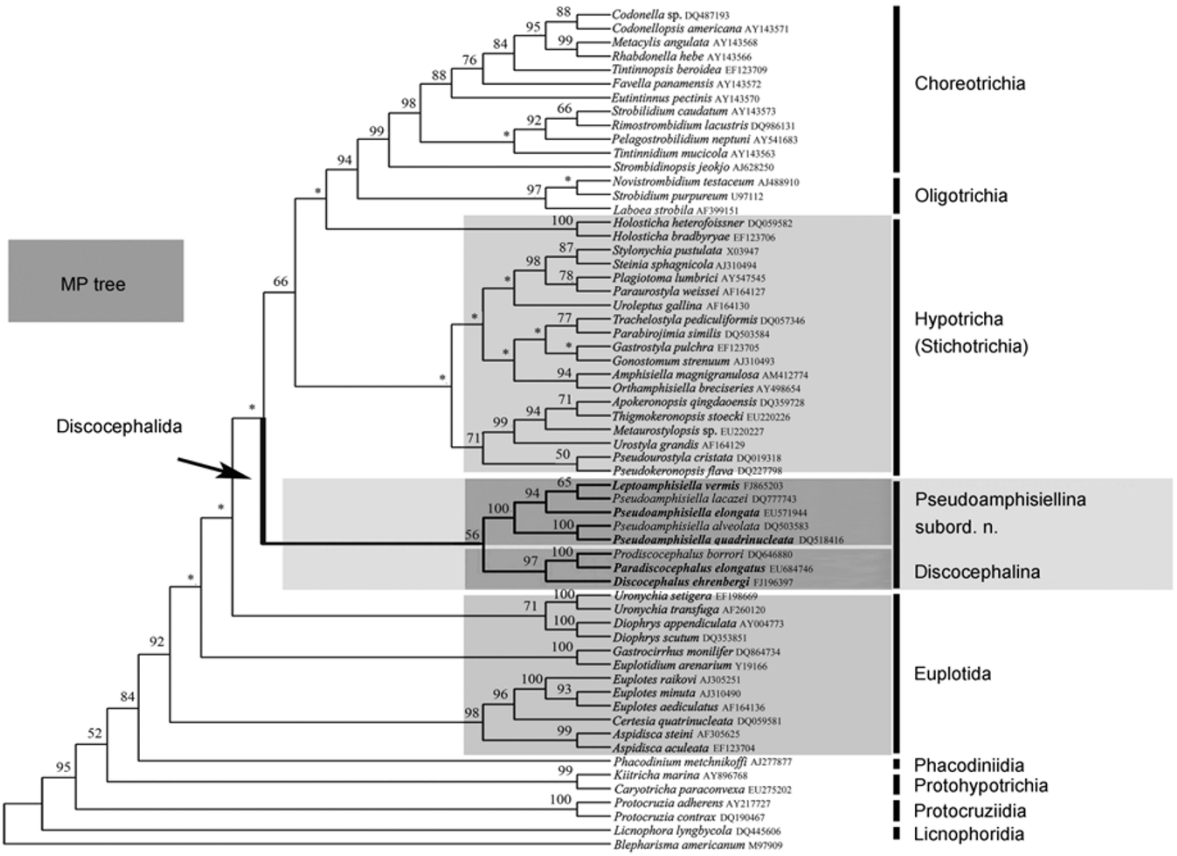

Figure 1 Maximum parsimony (MP) phylogeny of spirotrichous ciliates constructed from complete SSU rRNA gene nucleotide sequences. The numbers at the forks indicate the percentage of times that specific branch pattern occurred in 1000 trees. No significance is placed on branch lengths connecting the species. The taxa sequenced in our study are highlighted in boldface. Asterisks indicate bootstrap proportions <50%.

phyletic; (ii) Discocephalida is assigned to the hypotrichs; (iii) Discocephalida is placed within euplotids; (iv) *Leptoamphisiella* clusters with *Pseudoamphisiella*; and (v) the genus *Pseudoamphisiella* is monophyletic. The first (P= 0.078), third (P=0.122), and fourth (P=0.122) topologies were accepted, but the other topologies were strongly rejected (i.e., for 2, P=0.022; for 5, P=0.004).

3 Discussion

3.1 *Discocephalida* is clearly separated from euplotids and hypotrichs

Jankowski [28] placed the discocephalids as a family under the superfamily Oxytrichidea, and thus completely separated from euplotids. Based mainly on morphological features, the *Discocephalus*-like assemblage has been placed as follows: within the order Euplotida as a suborder along with the suborder of 'true' euplotids [3,29]; assigned to the family Euplotidae [1]; as a suborder within the order 'Hypotrichida' (s. l.) [5,30]; or as an order within Oxytrichia [31] or in the order Hypotrichida [5].

The morphological and morphogenetic data obtained to date reveal that the discocephalines possess a combination of features that are characteristic of either hypotrichs or euplotids (Table 1). In relation to most hypotrichs, discocephalines share the following features: (i) Both left and right marginal rows are present and formed intrakinetally, which is typical of most hypotrichs; (ii) the oral primordium in the opisthe is generated on the cell surface, hence in epi-apokinetal mode; (iii) the left-most frontal cirrus derives from the anterior end of the undulating membrane (UM)-anlage; (iv) many frontoventral-transverse (FVT) cirral anlagen are formed, which is widely regarded to be a plesiomorphic feature shared typically by the "lower" hypotrichs, and hence not the 5-FVT-mode seen commonly in euplotids; and (v) the dorsal kinety anlagen are formed in the secondary mode [9,11,12,32]. Discocephalines show also some features characteristic of typical euplotids: (i) the caudal cirri are formed from the rightmost dorsal kineties anlagen with a multi-segmentation mode; and (ii) the development of the FVT-anlagen is of the primary type, although this feature also occurs in some lower hypotrichs [10–12,33–35]. Nevertheless, considering the developmental mode and process, discocephalines are also rather unusual,

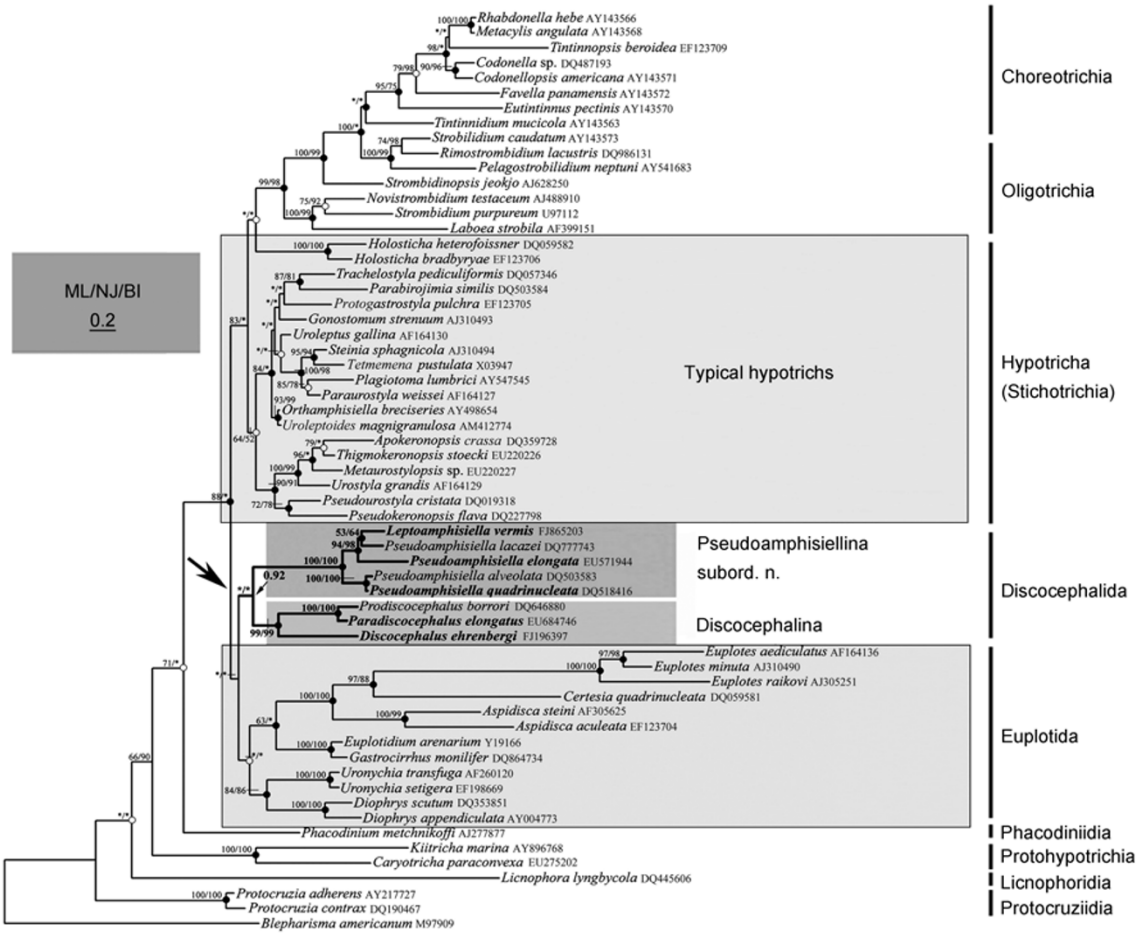

Figure 2 Maximum likelihood (ML) phylogeny of spirotrichous ciliates inferred from complete SSU rRNA gene nucleotide sequences. Numbers at the nodes represent bootstrap values (in %): $1P^{stP}$ No. = bootstrap values derived from ML method out of 1000 replications, $2P^{ndP}$ No. = bootstrap values derived from the distance matrix-based on the neighbor joining (NJ) method. Dots at nodes represent Bayesian posterior probabilities: a solid dot indicates 1.00 BI, and an open dot indicates 0.95–0.99 BI. Asterisks at a given node indicate bootstrap values less than 50% and/or disagreement between an analytic method and the reference ML tree. Evolutionary distance is represented by the branch length separating the species in the figure. The scale bar corresponds to 20 substitutions per 100 nucleotide positions. The taxa sequenced in our study are highlighted in boldface.

demonstrating features that occur in neither hypotrichs nor in euplotids: (i) migrating cirri are not formed, which are always derived from the right-most cirral anlage in all traditional hypotrichs; and (ii) the UM-anlage splits transversely to form the endoral and paroral membranes, whereas the UM-anlage typically splits longitudinally [5,9].

Our SSU rRNA gene-based analyses yielded two possible relationships, although both with low bootstrap values (<50%): The topology of the ML/NJ trees indicated a close relationship between the pseudoamphisiellid-discocephalid clade and the subclass Euplotida (Figure 2); the MP topology indicated the clade to be an intermediate group, divergent from the assemblage of hypotrichs-choreotrichs-oligotrichs (Figure 1).

Therefore, a hypothesis to explain the confusing suite of morphological and morphogenetic characters that seems to be characteristic of euplotids/hypotrichs can be formulated by focusing on the apparently basal (or divergent) relationship to pseudoamphisiellid-discocephalid. Different structural and developmental features of organisms do not necessarily evolve at the same rate. Therefore, the morphological/morphogenetic similarities may be plesiomorphies, and the conflicting mixture of characteristics seen in discocephalids may derive from different rates of character evolution. This supports ranking it at least as an ordinal taxon, as suggested by Shao *et al.* [9].

In conclusion, all of the above morphological and morphogenetic characteristics and molecular data indicate that discocephalids might represent a taxon intermediate between euplotids and hypotrichs. Nevertheless, as the AU test rejects their placement with subclass Hypotricha and there are significant morphological and morphogenetic differences compared to other hypotrichs, we propose elevating the suborder Discocephalina to ordinal status within the subclass Hypotricha.

3.2 Relationships of *Discocephalus*, *Paradiscocephalus*, and *Prodiscocephalus*

Discocephalids have a distinct discoid “head” followed by

Table 1 Comparison of ontogenetic characteristics among three higher hypotrichous groups. Alv, *Pseudoamphisiella alveolata*; Asp, *Aspidisca*; AZM, adoral zone of membranelles; Diop, *Diophrys*; Dis, *Discocephalus*; DK, dorsal kineties; Elon, *Pseudoamphisiella elongata*; Eup, *Euplotes*; FVT-anlagen, frontoventral-transverse cirral anlagen; Lac, *Pseudoamphisiella lacazei*; Mar, *Marginotricha*; Prod, *Prodiscocephalus*; UM, undulating membranes; Uron, *Uronychia*[a)]

Characteristics	Hypotrichs	Euplotids[§]	Pseudoamphisiellines	Discocephalines
Fate of old AZM	completely retained[&]	completely retained (Eup & Asp)	completely renewed (Lac & Elon)	completely retained (Dis)
	partly or completely renewed[†]	partly renewed (Diop & Uron)	partly renewed (Alv)	partly renewed (Mar & Prod)
Fate of old undulating membranes	completely renewed	retained (Eup & Asp) completely renewed (Diop & Uron)	completely renewed	completely renewed
Origin of the opisthe's oral primordium	on cell surface	within a subsurface pouch, beneath the pellicle	on cell surface	on cell surface
Origin of the left-most frontal cirrus	typically from UM-anlage	formed *de novo* near the old UM	from UM-anlage	from UM-anlage
FVT-cirral anlagen, number	both 5- anlagen[&] and more than 5[†]	invariably 5-anlagen	more than 5	more than 5
Type of development of	almost absolutely	primary mode	primary mode (Alv & Elon)	primary mode
FVT-anlagen	secondary mode[&]		secondary mode (Lac)	
Marginal anlagen	mostly intrakinetally less de novo	*de novo*	intrakinetally	intrakinetally
Dorsal kinety anlagen	mostly two-group type[#&] less commonly one group[†]	one-group type	one-group type	one-group type
DK formation mode[*]	secondary	primary	secondary	secondary
Origin of dorsal kineties	within left parental rows, followed by fragmentation in right-most row[&] intrakinetally or *de novo*[†]	within each of parental one, no fragmentation	within each of parental one, no fragmentation	within each of parental one, no fragmentation
Formation of caudal cirri	**	right-most one(s) generating one or more caudal cirri (euplotid mode)	**	***
Data sources	[12,13]	[33,34,36,37]	[32,38]	[5,9]

a) &, basically genera in the oxytrichid complex (s. l.). †, typical in urostylids. §, including genera for which morphogenesis is well characterized (e.g., *Euplotes*, *Diophrys*, *Uronychia* and *Aspidisca*). #, one group generates intrakinetally from the parental dorsal kineties, the other is formed *de novo* dorsal-marginally. *, initially as one group (primary mode) and then divides into two sets, one for each divider; or initially as two groups for two daughter cells (secondary mode). **, when present, each dorsal kinety anlage generates only one caudal cirrus. ***, multi-segmentation mode.

the body region [3]. Considering the general morphology, three genera have been discussed recently. *Paradiscocephalus* was not only mainly characterized by common *Discocephalus*-like features (e.g., the discoid 'head', posterolateral marginal row, general ciliary pattern, and well-developed transverse cirri), but also by special pairs of cortical granules and by ventral cirri arranged in a sparse zig-zag pattern corresponding to pseudoamphisiellids. The genera *Discocephalus* and *Prodiscocephalus* are similar to *Paradiscocephalus*, but have a tiny difference in the arrangement of ventral cirri (several unaligned and widely separated ventral cirri vs. ventral cirri arranged in an indistinct zig-zag pattern) [6,39]. The systematic relationship of these three genera has never been examined previously using molecular data. Our analyses of SSU rRNA gene sequences show consistently that *Prodiscocephalus*, *Paradiscocephalus* and *Discocephalus* share a common ancestry (99% ML, 1.00 BI, 99% NJ, 97% MP). Our results also demonstrated that *Prodiscocephalus* is closer to *Paradiscocephalus*, which is not congruent with their morphology.

3.3 The family Pseudoamphisiellidae belongs to the order Discocephalida

The systematic position of the genus *Pseudoamphisiella* Song, 1996 and the family Pseudoamphisiellidae Song *et al*., 1997 has never been determined unequivocally. In their recent revisions, Berger [12] and Shao *et al.* [32] regarded this family as a peripheral group within the urostylids. Very recently, in their phylogenetic investigation, Yi *et al.* [7] suggested that the family Pseudoamphisiellidae should be transferred into the Discocephalida, which has yet to be defined but includes both the Pseudoamphisiellidae and the Discocephalidae. Morphologically, *Pseudoamphisiella* is considerably divergent from typical urostylid hypotrichs (e.g., *Holosticha*, pseudokeronopsids, and *Urostyla*) be-

cause the cirri of its midventral rows are arranged in a separate, non-zig-zag pattern, and especially as there are/is no migrating cirri/row (frontoterminal cirri/row) during morphogenesis, which are formed characteristically from the posterior-most (right-most) FVT-cirral anlage [40]. In addition, the caudal cirri, which are lacking in all typical urostylids, are formed uniquely from the posterior end of each dorsal kinety anlage and the right marginal row has a unique de novo origin (i.e., appears as an independent anlage neighboring other FTV-primordia vs. being developed always intrakinetally within the parental structure in all other known hypotrichs) [12,32,38,41–45]. All these features cast strong doubt on the classification of *Pseudoamphisiella*, and therefore the family Pseudoamphisiellidae, among the urostylid hypotrichs.

Based on SSU rRNA sequences, pseudoamphisiellids are revealed as the sister group to the well-known discocephalines in all phylogenetic trees, although not with strong support. Nevertheless, this topology is consistent with both morphological (e.g., cephalized body shape, having a highly developed fiber system that connect cirri, generally two clearly separated ventral rows, and highly developed transverse cirri; for details see Table 2 and Figure 3) and general morphogenetic characters (e.g., the unique formation of the ventral rows during morphogenesis, which is clearly different from that of other typical hypotrichs) (Table 1) [5,9,32]. All such similarities suggest that the families Pseudoamphisiellidae and Discocephalidae should be assigned to the same assemblage, the order Discocephalida. However, these families are distinctive based on morphological, morphogenetic patterns, and SSU rRNA gene sequences (Table 2, Figure 3).

3.4 Establishment of a new suborder: Pseudoamphisiellina subord. n.

According to the morphological, morphogenetic and molecular data, the family Pseudoamphisiellidae invariably represents a distinct evolutionary assemblage sufficiently divergent to warrant separation as a suborder within the order Discocephalida [5,6,9,32,38,44–46], thus a new suborder, Pseudoamphisiellina, is proposed.

3.4.1 Diagnosis of suborder Pseudoamphisiellina subord. n.

Slightly or non-cephalized Discocephalida with untypical midventral rows, that is, two distantly separated rows that morphogenetically derive with an urostylid pattern; the right-most midventral row formed from the anterior-most cirri by each FVT-anlage, positioned on the right side; no frontoterminal row present that is formed from the posterior-most FVT-cirral anlage during morphogenesis; marine habitat, psammophilic.

3.4.2 Type family Pseudoamphisiellidae Song et al., 1997

This new suborder is currently a monotypic taxon containing only the type family Pseudoamphisiellidae, in which two genera can be clearly assigned: *Leptoamphisiella* Li *et al.*, 2007 and *Pseudoamphisiella* Song, 1996. It differs from the sister suborder Discocephalina in the body shape (only slightly or non-cephalized vs. typically cephalized), conspicuously different ciliature pattern (presence of non-migrating ventral row vs. absence; marginal rows uniform, non-differentiated vs. conspicuously bipartite or reduced) and many morphogenetic features [5,9,32,44,45]. In addition, molecular data consistently indicate that both groups are distinct and form a clearly defined assemblage [46].

Table 2 Morphogenetic and morphological characteristics used for assessment of phylogenetic relationships among representatives of discocephalids and other spirotrichous genera[a)]

Apomorph	Plesiomorph
1. Stomatogenesis in hypo-apokinetal mode	in epi-apokinetal mode
2. New OP will be formed in the proter	no new OP formed in the proter
3. UM in proter coming from de novo formed isolated UM-anlage	UM-anlage formed from dedifferentiated old structure
4. Cirral formation in 5-anlagen-mode, having 5-TC	in non-5-cirral-anlagen mode
5. Formation of caudal cirri in euplotid mode*	in normal stichtrichous mode
6. Right marginal row absent	present
7. DK anlagen not formed in each old row, often grouped	formed intrakinetally in each old row
8. One marginal row on each side	more than one on each side
9. Dorsal kineties formed in secondary mode	in primary mode
10. FVT-cirral anlagen formed in secondary mode	in primary mode
11. Having two undulating membranelles	only one undulating membrane
12. Cell with a 'head' or cephalized	non-cephalized
13. Ma nodules not fusing into a single mass during cell division	fusion into a single mass
14. No mid-ventral rows	present
15. Number of ventral cirral streaks very stable	variable in number

a) See Figure 3. Data sources: [5,6,9,11,12,32,33,37,38,40,43,47–51]. Abbreviations: DK, dorsal kineties; FVT, frontoventral-transverse; Ma, macronuclear; OP, oral primordium; TC, transverse cirri; UM, undulating membrane. *, This concerns the basic situation in which caudal cirri developed from the posterior end of right-most one or several dorsal kineties.

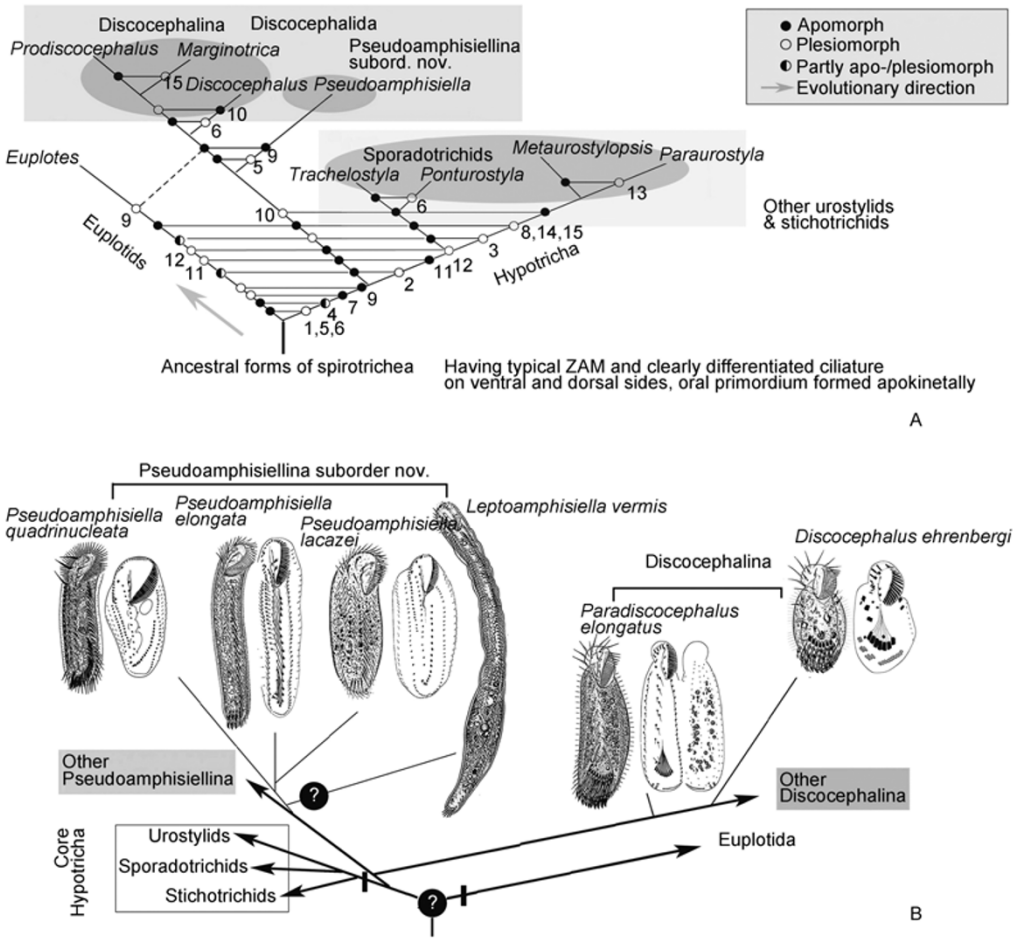

Figure 3 Assessment of the phylogenetic relationships among discocephalids and some other representative hypotrichs-euplotids based on morphological and morphogenetic information (A and B) (for explanation of numbered characters, see Table 2). Drawings of the representative forms that are analyzed in the present work are according to previous studies [6,43,45,50] (with permission of the respective authors).

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